Activity of taurolidine gels on ex-vivo periodontal biofilm

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Abstract

Objectives**:** To evaluate the activity of two different taurolidine (TAU) gels in comparison with a 0.2% chlorhexidine (CHX) gel on an ex-vivo subgingival biofilm.

Material and Methods: Subgingival incl. supragingival biofilm samples from periodontitis patients were cultured for 10 d, before TAU 1% and TAU 3% gels and CHX gel were applied for 10 min and thereafter diluted with nutrient media to 10% for 50 min. One third of the samples was analyzed for bacterial counts, biofilm quantity and biofilm metabolic activity. In the two other thirds, 90% of the nutrient media were replaced and biofilms were incubated for 23 h. The second third was analyzed in the same way as before. In the third part patients' microorganisms were added again and incubated for additional 24 h to allow reformation of biofilm before proceeded to analysis.

Results: Decrease of bacterial counts in biofilms was highest following application of TAU 3% after 60 min (0.87 log10 cfu, corresponding 86.5%), 24 h and 48 h (reformation of biofilms), respectively. All antimicrobials reduced biofilm quantity after 24 h (each $p<0.05$) and following reformation of biofilms (each $p<0.01$). Metabolic activity in biofilms was decreased at 60 min (each $p<0.05$) and at 24 h (each $p<0.01$) after application of TAU gels, while the activity of the reformed biofilm was lower after application of all evaluated antimicrobials (each $p<0.01$) than in the control group (e.g. without exposure to antimicrobials).

Conclusion: The antimicrobial activity of taurolidine gels clearly depends on its taurolidine concentration. A high concentrated taurolidine gel is equally active or even superior to 0.2% chlorhexidine gel. However, the activity of antimicrobials is limited in a complex established biofilm and underlines the pivotal role of mechanical biofilm disruption.

Clinical relevance: Within their limits, the data suggest that TAU 3% gel might represent a potential alternative to 0.2% chlorhexidine gel.

Key words: biofilm; antimicrobial; periodontal therapy

Introduction

Periodontitis is an inflammatory oral disease in response to oral biofilm which affects and destroys the tooth`s supporting tissues leading finally to tooth loss. The etiology of periodontitis is that microbiota in subgingival biofilm including *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola* induces innate, inflammatory and adaptive host response [1]. Certain microorganisms, e.g. *P. gingivalis* may act as key-stone pathogens by changing a symbiotic microbiota into a dysbiotic one via modifying host response [2].

The key step of periodontal therapy consists of the removal of supra- and subgingival bacterial biofilm [3]. A systematic review showed that improvement of clinical attachment level is primarily achieved by mechanical non-surgical therapy [4], the use of antimicrobials are generally recommended for additional use [5]. In dentistry, chlorhexidine is one of the best documented antimicrobial agents [6]. Chlorhexidine is active against most microbial species [7, 8], but it is also cytotoxic [9, 10]. Moreover, discoloration, taste irritation occur frequently after application of various chlorhexidine-containing formulations [11, 12]. Substantial data indicate that the use of adjunctive systemic antibiotics appears to be beneficial in advanced and severe cases to arrest the bacterial biofilm caused infection and subsequent inflammation [13]. However, the long-term clinical benefit following the use of antibiotics is still unclear and needs to be carefully considered, especially in the light of the global increase of antibiotic resistance [14, 15]. Therefore, the use of systemic antibiotics in the treatment of periodontal infections should be carefully considered and obviously, there is a clear need for alternative antimicrobial agents.

Taurolidine is most discussed as a lock solution for central venous catheters because of its antibacterial and antibiofilm properties [16]. Antimicrobial activity was described against gram-positive and gram-negative bacteria as well as against *Candida* spp. [17]. It exerts its activity by inactivating endotoxin in gram-negative bacteria [18] and interacting with peptidoglycan [19].

Taurolidine appears to be a potential alternative also in dentistry. Several years ago, a potential antimicrobial activity of taurolidine was reported against oral microorganisms [20, 21]. Recently, our group has shown that 2% taurolidine is effective in killing supragingival plaque [22]. In earlier studies, the MICs of taurolidine against oral species were determined as all below 5% of the normally used concentration of that substance with the exception of *Candida albicans* [23]. Moreover, previous data from a series of studies performed in our laboratory

have shown that taurolidine solutions and taurolidine gels inhibited clearly the formation of defined biofilms containing laboratory strains [24, 25].

Therefore, the aim of the present study was to determine the activity of two different taurolidine gels as compared with a 0.2% chlorhexidine gel on an ex-vivo subgingival biofilm formed for 10 d and on its reformation including the assessment of the killing of bacteria within the biofilm, of the biofilm quantity and of the biofilm metabolic activity.

Material and methods

Sampling from chronic periodontitis patients

The Ethical Committee of the Canton Bern approved the study protocol (KEK 035/2015). After signing the informed consent form, biofilm samples were obtained from nine patients with advanced chronic periodontitis referred to the Department of Periodontology, University of Bern, School of Dental Medicine.

For inclusion in the study, the following criteria had to be fulfilled: probing depths (PD) of ≥5 mm at least at four non adjacent sites and presence of at least two of the main four bacterial species associated with periodontal disease: *A. actinomycetemcomitans, P. gingivalis*, *T. denticola* and *T. forsythia* detected via routinely used microIdent® test (Hain Lifescience, Nehren, Germany). Exclusion criteria were: intake of antibiotics three months prior to the study, periodontal therapy within the last six months, diabetes or other severe systemic disorders affecting the immune system.

Patients were asked to refrain from oral hygiene for 24 h in the areas where plaque was to be sampled. The deepest pockets per quadrant were selected. Without removing supragingival plaque each two paper-points (ISO 055) were inserted until resistance was felt. After 30 s, the paper points were removed, transferred into tubes containing 1 ml of transport media (reduced transport fluid (RTF)) [26], and proceeded immediately to the laboratory where they were kept at 4°C

In addition, about 2 ml of non-stimulated saliva were collected from each individual and centrifuged at 500 *g* for 10 min.

Antimicrobials

The antimicrobials to be tested were taurolidine in 1% (w/w) and 3% (w/w) gel formulation (1% PerioSept Gel, 3% PerioSept Gel, both Geistlich Pharma AG, Wolhusen, Switzerland).

A 0.2% (w/w) chlorhexidine gel (Plak-Out Gel (Kerr-Hawe SA, Bloggio, Switzerland) as a positive control and dH_2O as a negative control were be included in addition.

Ex-vivo biofilm model

The ex-vivo model was modified to that described by Walker & Sedlacek [27].

Biofilms were cultured for each of the nine included patients separately. The day before starting biofilm formation, wells of 96-well plates (24-well plates containing glass slides for live-dead staining) had been prepared by coating with poly-L-lysine (1 : 10 diluted with dH_2O) overnight. After removing the non-attached poly-L-lysine solution, supernatants of the patient's own saliva mixed 1 : 1 with phosphate buffered saline (PBS) containing 50% of inactivated human serum (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) (10 µl 96-well-plate; 55 µl 24-well plate) were added and exposed to UV for 30 min and left in place. Meanwhile bacteria were suspended in RTF by vortexing and ultrasonication. An amount of 0.5 ml of RTF with bacteria was mixed with 19.5 ml of nutrient broth (Wilkins Chalgren broth (Oxoid, Basingstoke, GB) containing 5 µg/ml NAD and 5 µg/ml thiamine pyrophosphate (Sigma-Aldrich Chemie GmbH). Thereafter, 150 µl (1000 µl when using 24-well-plates) of the suspension was added to each well. Biofilms were incubated for 10 d in an anaerobic atmosphere (85% N_2, 10% H_2, 5% CO_2 in an anaerobic workstation (DG250, Don Whitley Scientific Ltd., Shipley, UK). Nutrient broth was exchanged every second day.

After careful removing the nutrient media, 20 µl (110 µl when using 24-well-plates) of the undiluted test substances (concentration of 100%) were given to biofilms. After 10 min, nutrient broth was added in a ratio 1 : 9 leading to a concentration of 10% of the substance in the media. After 60 min, media was removed and the biofilms were carefully rinsed in one third of the wells. Here, counts of bacteria, metabolic activity of biofilm, live-dead ratio and biofilm quantity (amount of matrix) were determined thereafter.

In the two other thirds of the wells, 90% of the nutrient media were exchanged by fresh ones (concentration of 1% of the substance in the medium). After an additional incubation of 24 h, procedure (without live-dead ratio) was as after 60 min in the second third.

From the last third of wells, the nutrient media was removed. After careful washing, 0.5 ml of RTF with bacteria (which were kept at -80°C) mixed with 19.5 ml of nutrient broth were added for an additional incubation of 24 h to assess the antimicrobials' influence on reformation of biofilm (recolonization). Thereafter at 48 h, samples were analyzed as for the time-point 24 h.

Analyses of biofilms

Bacterial counts were determined by enumeration of total colony forming units (cfu) after scraping the biofilm from the surface, extensive mixing, making a serial dilution and plating 25 µl each on tryptic soy agar plates with 5% of sheep blood and anaerobic cultivation for 10 d. In addition, 16S rDNA analysis (real-time PCR) was made for the presence of major species being associated with periodontitis (*P. gingivalis*, *T. forsythia*, *T. denticola* and *A. actinomycetemcomitans*) by using GoTaq qPCR Master Mix (Promega Corporation, Madison, WI, USA), as described recently [28].

Quantification of the biofilms was made according to recently published protocols [29]. After rinsing, the biofilms were fixed at 60°C for 60 min. Thereafter, biofilms were stained with 50 µl per well 0.06% (w/v) crystal violet (Sigma-Aldrich Chemie GmbH) for 10 min and the staining was quantified by using a plate reader (ELx808, Biotek Instruments, Winooski, VT, USA) at 600 nm.

Biofilm metabolic activity was assessed with using Alamar blue as a redox indicator [30]. Five µl of Alamar blue (alamarBlue® reagent, Thermo Fisher Scientific Inc., Waltham, MA, USA) was mixed with 100 µl of the nutrient media and added to the biofilm. After extensive mixing with the biofilm and an incubation for 1 h at 37°C, ratio of absorbances at 600 nm and 570 nm were calculated after measuring by using a microplate reader (ELx808, Biotek).

Biofilms formed on glass slides in 24-well-plates and proceeded as described above (treatment with antimicrobials, rinsing), were stained and visualized with live-dead staining (Live/dead® *Bac*Light™ Bacterial Viability kit, Invitrogen Corporation, Carlsbad, CA, USA) by using a fluorescent microscope (Olympus BX51, Tokyo, Japan) with the settings excitation 460-495 nm, emission 510-550 nm, mirror 505 nm for Cyto 9 dye and excitation 530-550 nm, emission 575-625 nm, mirror 570 nm for propidium iodide. Ratio between live and dead bacteria was related to the means of green or red color mean intensity by using Adobe® Photoshop@ Elements 9.0 software (Adobe Systems, Mountain View, CA, USA). This analysis was made only at the time-point 60 min.

Statistical methods

Biofilm samples per patient were always assayed in independent duplicates, meaning in a total 18 samples were analyzed per group each. ANOVA with Post-Hoc LSD was used for statistical analysis. The level of significance was set to p=0.05. Software SPSS 22.0 (IBM SPSS Statistics, Chicago, IL, USA) was used.

Results

Microbial counts

The cfu counts in biofilms without exposure to antimicrobials were 7.68±0.19 log10 cfu after 10 d (60 min), 7.88±0.25 log10 cfu after 11 d (24 h) and 8.60±0.61 after 12 d (48 h, 24 h of reformation of biofilm; p<0.001 vs. 60 min, 24 h). Sixty minutes after exposing the biofilms to any of the antimicrobials, the counts in biofilms were significantly reduced (TAU 1%: reduction 0.36 log 10 cfu, p=0.021; TAU 3%: reduction 0.87 log10 cfu, p<0.001 and CHX 0.2%: reduction 0.64 log 10 cfu, p<0.001), however the difference was at the highest 0.87 log10 cfu (TAU 3%) (corresponding to 86.5% reduction). TAU 3% was significantly more active than TAU 1% (p=0.001). After 24 h exposure, differences to control were still significant for TAU 3% (-0.58 log10, p<0.001) and for CHX 0.2% (-0.47 log10, p=0.001). Only TAU 3% was able to inhibit significantly reformation of biofilms (difference: -0.61 log10, p=0.004) (Fig. 1).

Samples being initially tested positively for the analyzed species were included in the nucleic-acid based analysis of single species. *P. gingivalis* was detected in seven, *A. actinomycetemcomitans* in three and *T. denticola* in two of the nine patients. All biofilm control samples had been positively tested for *T. forsythia*. Counts of *P. gingivalis* and *T. denticola* were reduced after 60 min exposure to TAU 3% (p=0.036, p=0.022) and CHX 0.2% (p=0.020, p=0.023). In reformed biofilm the counts were less for *T. forsythia* after TAU 3% (p=0.017) and for all analyzed species after CHX 0.2% (*P. gingivalis*: p=0.022, *T. forsythia*: p=0.039, *T. denticola*: p=0.003, *A. actinomycetemcomitans*: p=0.012) than in the controls (Fig. 2).

The live/dead ratio 60 min after antimicrobial exposure showed a decrease after CHX 0.2% treatment compared to control (p=0.006) and to TAU 1% (p=0.011; Fig. 3).

Biofilm quantity and metabolic activity

All treatments significantly reduced biofilm quantity after 24 h (TAU 3%: p=0.023, TAU 1%: p=0.028, CHX 0.2%: p=0.012) and after 48 h (reformation of biofilms for 24 h) (each p<0.001). There was no difference between the antimicrobials (Fig. 4).

Metabolic activity in biofilms was significantly reduced 60 min after TAU 3% (p=0.016) and TAU 1% (p=0.026) and 24 h after TAU 3% (p=0.002) and TAU 1% (p=0.004). The metabolic activity of the reformed biofilm was always lower after any antimicrobials application than in the control without exposure to antimicrobials (TAU 3% and TAU 1%: p<0.001, CHX 0.2%: p=0.001; Fig. 5)

Discussion

The purpose of the present in vitro study was to evaluate the activity of taurolidine gels on an ex-vivo biofilm generated from periodontitis patients' samples.

Samples including primarily subgingival but also supragingival biofilm were obtained from periodontitis patients that harbored bacteria associated with periodontal disease. Sampling method was in accordance with those used for routine microbiological analysis. It is based on a report that without removing supragingival biofilm the detection rate of bacterial species being associated with periodontitis is higher [31]. Subsequently, the plaque samples were used to form an ex-vivo biofilm. The modifications of the model described by Walker & Sedlacek [27] include the use of native saliva without any sterile filtration and the repeated addition of subgingival plaque samples to mimic reformation. The rationale for using native saliva is to ensure its unchanged protein composition, since it has been shown that filtering decreases the protein content and modifies the protein composition [32]. The results revealed consistently higher bacterial counts and higher biofilm quantity and metabolic activity in the reformed than in the first formed biofilm, thus confirming successful incorporation of these microorganisms in the biofilms.

In the present study a complex biofilm was formed and treated with antimicrobials. Subsequent analyses were performed by several methods. Ex vivo oral biofilms were occasionally used for determining activity of antimicrobials. Four hours – 10 days old biofilms had been exposed to antibiotics for 48 h [33]. The activity of chlorhexidine was compared with photodynamic therapy

on biofilms cultured for 24 h and 72 h [34]. Oral antiseptics (*e.g.* mouth rinses) were tested to inhibit salivary biofilm formation [35]. Sometimes, plaque samples are harvested and directly exposed to potential antimicrobials in the laboratory [36, 37].

Teeth are surrounded by a continuous flow of gingival crevicular fluid [38]. In order to simulate the in vivo situation, the applied antimicrobial was diluted to 10% after 10 min and in part additionally after 60 min to 1%. The highest reduction of bacterial counts after the application of antimicrobials was 86.5%. However, a killing activity of an antimicrobial is generally defined as a reduction by at least 3 log10 over a defined time, here only 0.87 log_{10} were reached. In our previously performed study using a defined 12-species biofilm, the same antimicrobial (TAU 3%) reached 3.63 $log_{10,}$ [25]. There 3% taurolidine has been in contact with the biofilm for 60 min before diluting to 0.3%, in the present study dilution was already after 10 min to 0.3%.

Comparing TAU 3% with TAU 1% a clear concentration dependent activity was visible on total bacterial counts (cfu) as well as on selected species (*P. gingivalis* and *T. denticola* after 60 min) in biofilm. This is in agreement with findings from previous studies using a 12-species biofilm, where higher concentrations of taurolidine solutions [24] and taurolidine gels [25] were more bactericidal than lower ones. However, in the present study, there was no difference between the two taurolidine gels regarding biofilm quantity and metabolic activity. Both gels decrease biofilm metabolic activity already after 60 min, thus suggesting that a lower concentration of taurolidine may already inhibit bacterial metabolism. Biofilm quantity determined by crystal violet staining is reduced after 24 h. Biofilms are not only bacteria, they consist of self-produced matrix of extracellular polymeric substances [39]. Interference with bacterial matrix components is an approach in the development of anti-biofilm drugs [40]; investigating this aspect in more detail might be of interest in further research. An activity of the vehicle used in taurolidine gels on biofilm metabolic activity and biofilm quantity cannot be ruled out. In our previous study [25] no influence of the vehicle on bacterial counts was seen. Due to the complexity of the study design, a vehicle could not be included.

In the present study, TAU 3% reduced as CHX 0.2% total bacterial counts (cfu) in biofilm after 60 min and 24 h. Although the reduction of percentage of viable bacteria related to dead bacteria failed to show statistical significance for TAU 3% in contrast to CHX 0.2%, there was no statistical significant difference between the two antimicrobials. TAU 3% was the only tested compound showing a very small, but statistically significantly different reduction of total bacterial counts (cfu) in the reformed biofilm. These findings are of particular interest, especially in comparison with chlorhexidine, a compound with known high substantivity [41].

Taken together the available data indicate that taurolidine is a potential alternative as an antimicrobial. It interacts with bacterial cell wall components [19] while in long-term users no decreased susceptibility of bacteria causing blood-stream infections was observed [42]. Beside of its antimicrobial activity taurolidine inhibits the expression of pro-inflammatory cytokines [43]. In an animal model there was no toxicity found [44], in in-vitro assays cytotoxicity was comparable to other agents like 3% hydrogen peroxide [45] or chlorhexidine [46].

However, the number of studies investigating the antibacterial properties of taurolidine on oral biofilms is still low. Besides the few studies focusing on supra- and subgingival biofilm [22, 24, 25] it was found, that in contrast to chlorhexidine, the antimicrobial activity of taurolidine is not affected in a serum rich environment [23]. Furthermore, a recent in vitro study showed, that taurolidine might enhance effectiveness of plaque removing procedures on titanium surfaces with plastic curettes and glycine powder airflow [47]. Interestingly, in simulated endodontic infections, taurolidine was more bactericidal than chlorhexidine but less than calcium hydroxide against *Enterococcus faecalis* biofilm [46].

All these findings appear to suggest that taurolidine may be a potentially relevant antimicrobial agent for an adjunctive use to scaling and root planning in the treatment of periodontal pockets. Since none of the currently available instrumentation techniques can completely remove the supra- and subgingival biofilm [48], the use of adjunctive antimicrobial substances may be of potential benefit [49]. Nevertheless, it should be kept in mind that in the present in-vitro study, the reduction of bacterial counts in biofilm never exceeded one log10 which in turn, underlines once more the fact that antimicrobials alone are not able to completely destroy an already established complex biofilm. Therefore, the use of antimicrobials can only be recommended in conjunction with mechanical biofilm disruption suggesting once more that mechanical biofilm removal is still the gold standard in the therapy of periodontitis [50].

In summary, the present study has shown that: (i) an ex-vivo biofilm model that closely resembles the in vivo situation and enables the evaluation of various antimicrobials can be predictably established; (ii) the activity of antimicrobials is limited in a complex established biofilm; (iii) the antimicrobial activity of taurolidine gels clearly depends on its active compound concentration; (iv) 3% taurolidine gel appears to be an interesting alternative to chlorhexidine as an adjunct in periodontal therapy and warrants further evaluation in clinical settings.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figure legends

Fig. 1 Total counts of colony forming units (cfu) in biofilm 60 min and 24 h as well as after 48 h (allowing reformation of biofilm for 24 h) after exposing biofilms to undiluted 0.2% chlorhexidine (CHX) gel or 1% and 3% taurolidine (TAU) gels for 10 min and a following dilution to 10% of the gels (0.02% CHX, 0.1% TAU, 0.3% TAU)
*p<0.05; **p<0.01 compared to control
**p<0.01 compared to TAU 3%
*p<0.05 compared to TAU 1%

Fig. 2 Counts of selected bacterial species in biofilm 60 min and 24 h as well as after 48 h (allowing reformation of biofilm for 24 h) after exposing biofilms to undiluted 0.2 % chlorhexidine (CHX) gel or 1% and 3% taurolidine (TAU) gels for 10 min and a following dilution to 10% of the gels (0.02% CHX, 0.1% TAU, 0.3% TAU)

*p<0.05; **p<0.01 compared to control
*p<0.05; **p<0.01 compared to TAU 1%

Fig. 3 Live/dead ratio in biofilms 60 min after exposing biofilms to undiluted 0.2 % chlorhexidine (CHX) gel or 1% and 3% taurolidine (TAU) gels for 10 min and a following dilution to 10% of the gels (0.02% CHX, 0.1% TAU, 0.3% TAU)

**p<0.01 compared to control
*p<0.05 compared to TAU 1%

Fig. 4 Biofilm quantity expressed in extinction units at 594 nm after crystal violet staining in biofilm 60 min and 24 h as well as after 48 h (allowing reformation of biofilm for 24 h) after exposing biofilms to undiluted 0.2 % chlorhexidine (CHX) gel or 1% and 3% taurolidine (TAU) gels for 10 min and a following dilution to 10% of the gels (0.02% CHX, 0.1% TAU, 0.3% TAU)

*p<0.05; **p<0.01 compared to control

Fig. 5 Biofilm metabolic activity expressed as ratios of extinction units at 595/570 nm in biofilm 60 min and 24 h as well as after 48 h (allowing reformation of biofilm for 24 h) after exposing biofilms to undiluted 0.2 % chlorhexidine (CHX) gel or 1% and 3% taurolidine (TAU) gels for 10 min and a following dilution to 10% of the gels (0.02% CHX, 0.1% TAU, 0.3% TAU)

*p<0.05; **p<0.01 compared to control

*p<0.05 compared to TAU 3%

*p<0.05 compared to TAU 1%

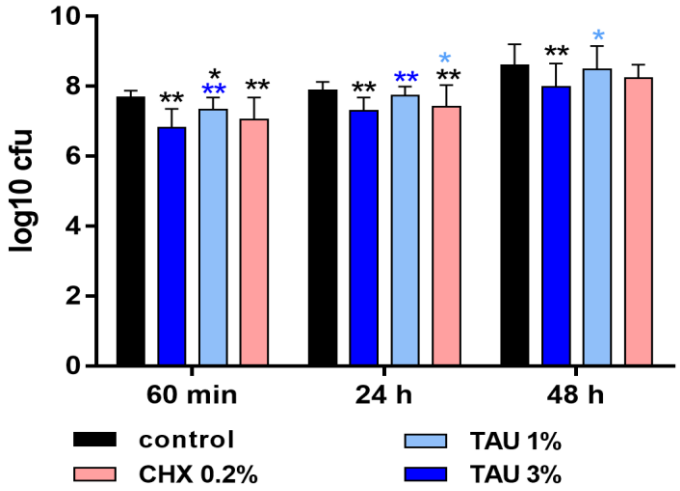

Figure 1

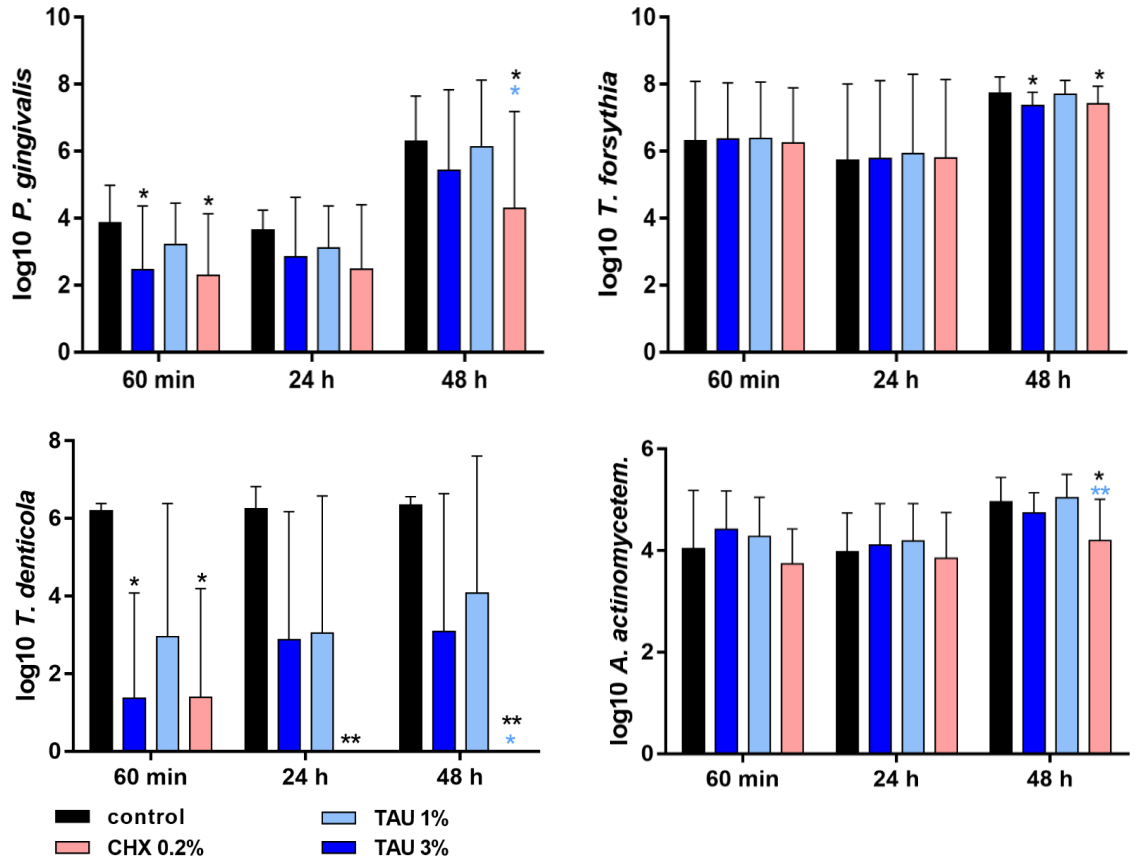

Figure 2

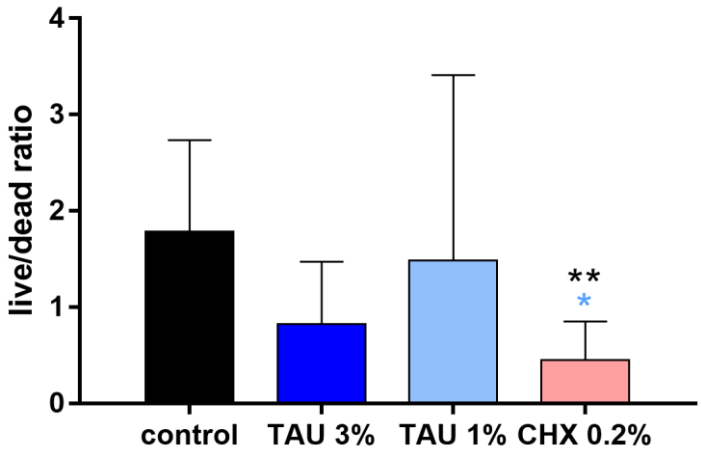

Figure 3

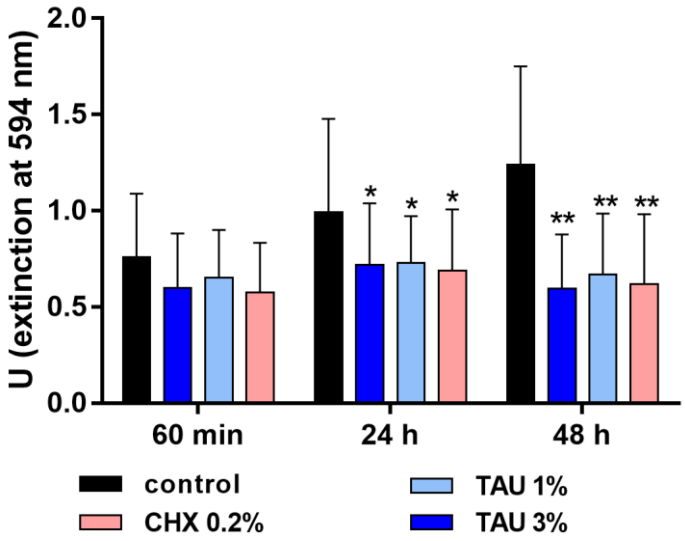

Figure 4

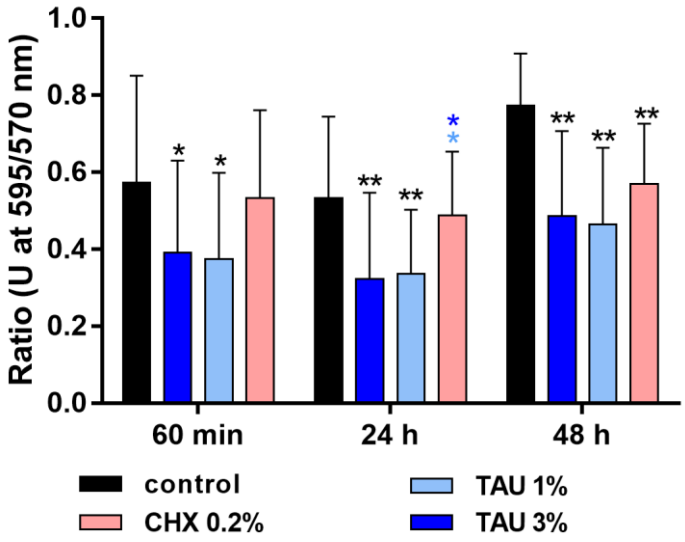
Ratio (U at 595/570 nm)
1.0
0.8
0.6
0.4
0.2
0.0
60 min
24 h
48 h
control
CHX 0.2%
TAU 1%
TAU 3%

Figure 5